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File No. / Attorney **22908-0002 PM/njm**

Application No. __ **08/857,389**

Date Mailed _____ **September 16, 1999**

Documents _____ **Transmittal (in duplicate);**

_____ **Declaration of J. Gregor Sutcliffe, Luis de Lecea, Steven J. Henriken, and George R. Siggins Under 37 C.F.R. § 1.131;**

_____

Certificate of Mailing Under 37 C.F.R. § 1.8

OIPE JC79 SEP 21 1999 PATENT & TRADEMARK OFFICE

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Atty. Docket No. 22908-0002
Client Ref. TSRI519.1/SCR1919P

CERTIFICATE OF MAILING PURSUANT TO 37 C.F.R. § 1.8

I hereby certify that this papers is being deposited in the United States mail as first class mail with postage prepaid, and is addressed to: Assistant Commissioner for Patents, Washington D.C. 20231, on September 16, 1999 in Palo Alto, CA.

N J Miller

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of)
Sutcliffe et al.)
)
For: **CORTISTATIN: NEUROPEPTIDES, COMPOSITIONS AND METHODS**)
)
Serial No.: 08/857,389)
)
Filed: May 15, 1997)

Examiner: R. Hayes

Group Art Unit: 1645

TRANSMITTAL

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Transmitted herewith for filing in the above-identified patent application is the Declaration of J. Gregor Sutcliffe, Luis de Lecea, Steven J. Henriksen, and George R. Siggins Under 37 C.F.R. § 1.131, and a Return Receipt Postcard.

No fee is due with this communication. The Commissioner is hereby authorized to charge any fees and credit any overpayment of fees which may be required under 37 C.F.R. § 1.16 and § 1.17, to Deposit Account No. 08-1641, referencing Atty. Docket No. 22908-0002.

By: Priscilla Mark

Priscilla Mark
Attorney for Applicants
Registration No. 41,970

Heller Ehrman White & McAuliffe
525 University Avenue, Suite 1100
Palo Alto, CA 94301-1900
Direct Dial: (650) 324-7184
Facsimile: (650) 324-0638

CERTIFICATE OF MAILING PURSUANT TO 37 CFR 1.8
I hereby certify that this papers is being deposited in the United States mail as first class mail with postage prepaid, and is addressed to: Assistant Commissioner for Patents, Washington D.C. 20231, on 9/16/99 in Palo Alto, CA.

N J Miller

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of	)	Examiner: R. Hayes
J. Gregor Sutcliffe *et al.*	)	Group Art Unit: 1645
For: **CORTISTATIN: NEUROPEPTIDES, COMPOSITIONS AND METHODS**	)	
Serial No.: 08/857,389	)	
Filed: May 15, 1997	)	

DECLARATION OF J. GREGOR SUTCLIFFE, LUIS DE LECEA, STEVEN J. HENRIKSEN, AND GEORGE R. SIGGINS UNDER 37 C.F.R. § 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

We, J. Gregor Sutcliffe, Luis De Lecea, Steven J. Henriksen, and George R. Siggens, hereby declare that:

1. We are inventors in the above identified application.

2. We conceived and reduced to practice the invention claimed in the above identified application in the United States prior to March 6, 1997.

3. We isolated and purified mammalian cortistatin, namely rat, mouse, and human cortistatin, and genes encoding the mammalian cortistatin, prior to March 6, 1997.

4. On information and belief, acting on our direction, Patricia E. Danielson and Pamela E. Foye, technicians for The Scripps Research Institute, the present assignee of the parent application for the above identified application, Serial No. 08/648,322, isolated fragments of the human cortistatin coding sequence using degenerate primers from rat sequences, made probes and screened a human whole brain cDNA library with the probes, and thereby isolated and purified DNA clones encoding human cortistatin, and made laboratory note book entries describing this work. Copies of the notebook entries of Patricia E. Danielson and Pamela E. Foye are attached hereto as Exhibit A, with the dates on the documents redacted.

5. On information and belief, and on first hand knowledge on the part of J. Gregor Sutcliffe, on a date prior to March 6, 1997, J. Gregor Sutcliffe sent a letter to William Schmonsees, who is outside patent counsel for The Scripps Research Institute, with a manuscript disclosing the invention. A copy of the letter and accompanying manuscript are attached hereto as Exhibit B, with the dates on the documents redacted.

6. The manuscript discloses the claimed isolated and purified mammalian, and specifically rat, mouse, and human, cortistatin, and genes encoding the mammalian cortistatin.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such false statements may jeopardize the validity of the above identified application or any patent issued thereon.

FIRST JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
J. Gregor Sutcliffe	J Gregor Sutcliffe	7/26/99

SECOND JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
Luis De Lecea	Luis de Lecea	8/2/99

THIRD JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
Steven J. Henriksen	Steven J. Henriksen	8/3/99

FOURTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
George R. Siggins	George R. Siggins	8/9/99

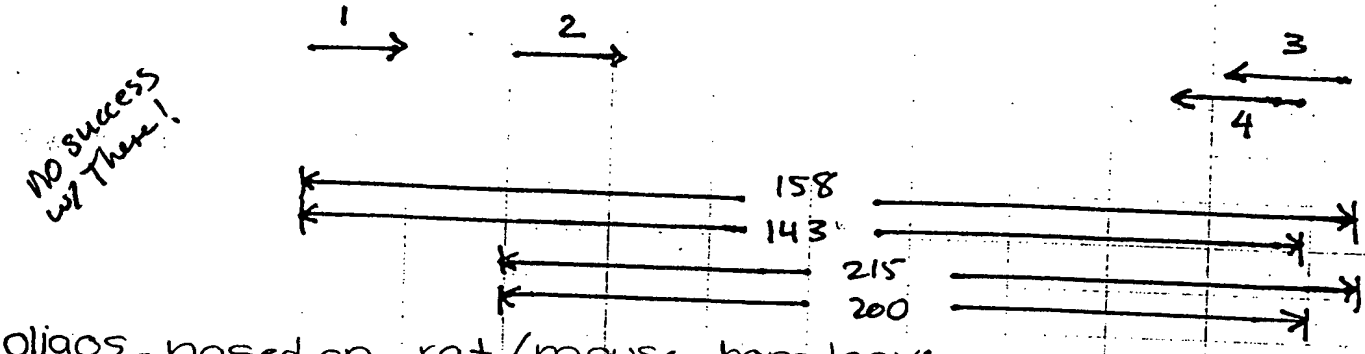
HEWM #157771

EXHIBIT A

H IAN

Cystatin - cloning project

Revisi
- Pa
- Pa



oligos - based on rat/mouse homology:

already made 1) C A A/G G A C/T T C A/C/G/T GT A/C/G/T CA A/G GA

"cst-two" 2) TGG TGG CA T/C G A G/A TGG

is "free" 3) C A A/G/C C G C/T A A/G C/T G A/G A A A/G/C/T G T C/T T TC

"cst-four" 4) TTCCA A/G A A A/G AA A/G T T T/C TT A/G CA

	Tm (ra
17mer	46 -5
15mer	46 -5
18mer	46 -5
20mer	46 -5

PCRs with human brain cDNA library

1. 1+3
2. 1+4
3. 2+3
4. 2+4
5. (1+3) & (1+4)
6. (1+3) & (2+3)
7. (1+3) & (2+4)
8. (2+3) & (2+4)
9. (1+4) & (2+4)

- P.F. trimmed down same cst-two & cst-three nucleotides- to make all 4 oligos have similar Tm °C
- P.D. double-checked these oligos

Pam Transformed the mixed miniprep of set controlling positive clones 11, 12 & 20 from ... She's spread plates on ... & grew out O/N.

- Picked 10 single colonies of each, resulting in 11-1 → 11-10, 12-1 → 12-10, & 20-1 → 20-10, and set up 5 ml LB+Amp O/N cultures.

Put away 100 µl of each prep w/ glycerol @ -70°C.

Alkaline lysis minipreps of 30 clones. Resuspended in 100 µl T.E. & stored @ -20°C O/W E.

Digest 5 µl of each w/ BamHI

5 µl DNA
5.3 µl dH_2O
1.2 µl 10×B buffer
0.5 µl BamHI

Mix — (35×)
185.5 µl dH_2O
42.0 µl 10×B buffer
17.5 µl BamHI
245 µl
→ 7 µl per tube.

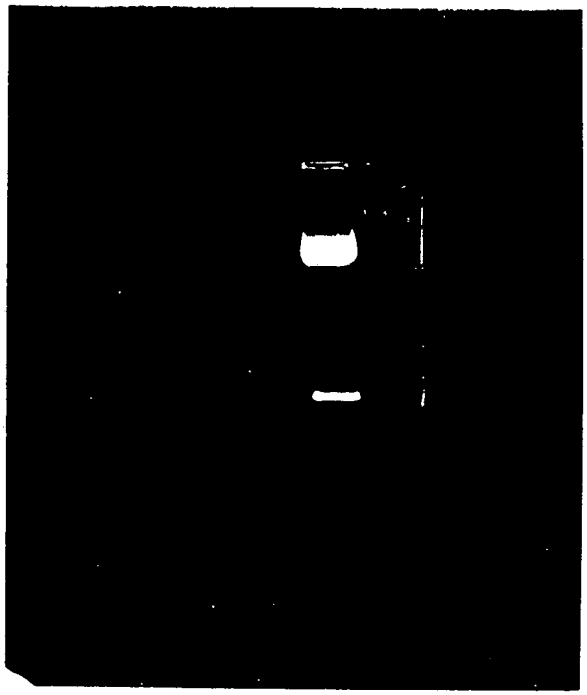
Aliquot 5 µl of each DNA sample + add 7 µl of enzyme digest mix. Incubate 1½ hrs @ 37°C.
Add 3 µl dye

Run on FMC 24 well format Seakem agarose gels in TBE

NB: no prospector.
Lifted filters from spread plates & probed w/ Express Hyb
Developed film on [illegible] picked +'s (?!)
+ put in to grow 3×50 ml O/N.

Cut 5 µg pBSK Cort – 0.8 µg/µl rat cortistatin ORF probe.

6.25	
14.25	
2.5	
1.0	
1.0	
25 µl	



Run on "old" LMP agarose gel from -stored wrapped @ 4°C.

Recover insert band.

Tare = 1.38
Rat Cort ORF = 1.44 - 1.38 = 0.06

(450/3450)5 = 652 ng

652 ng/60 µl = 11 ng/µl

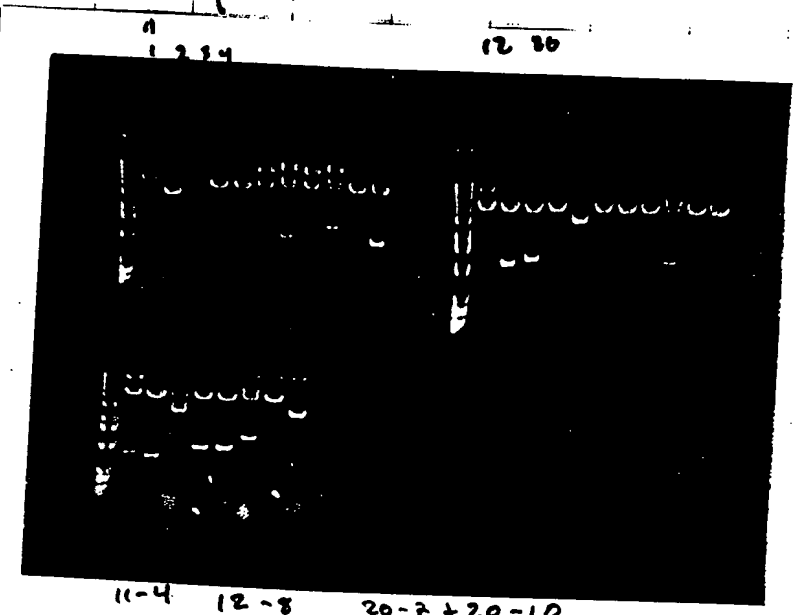
Use 4 µl for labeling

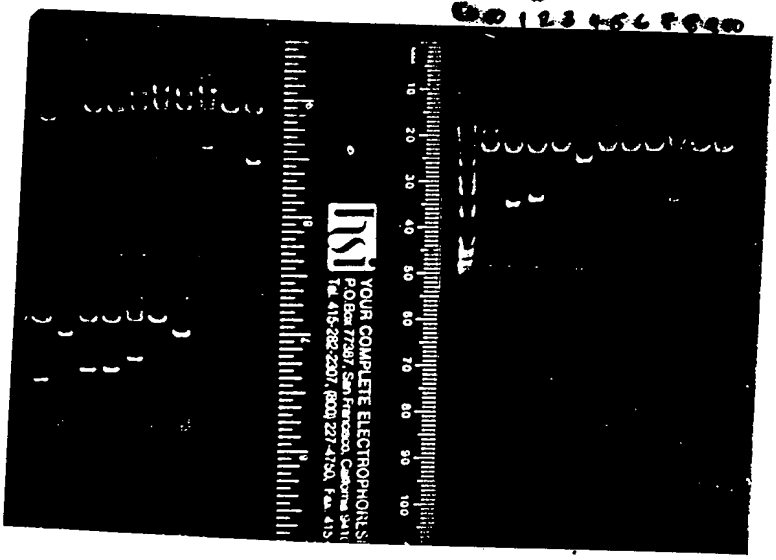
4 µl DNA
28 µl dH_2O
} 100°C for 7' → ~42°C

Add 1 µl BSA
10 µl 5X OCB
5 µl α^{32}P dCTP
2 µl Klenow (2U/µl)
50 µl

1 hr. @ 37°C
O/N @ RT.

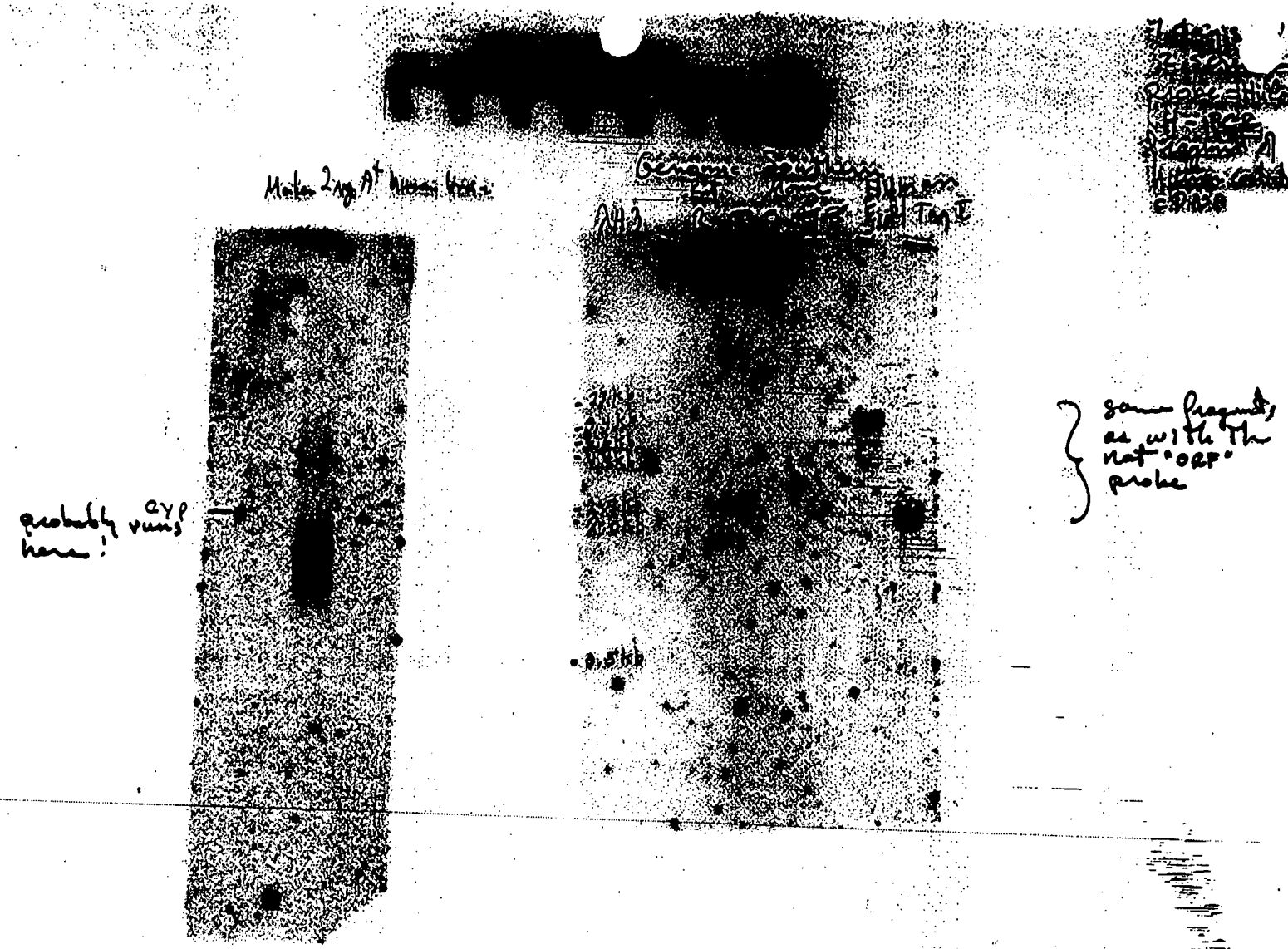
Photos of gels:





Putative positives:

11-4(s?)
12-8
20-7
20-10

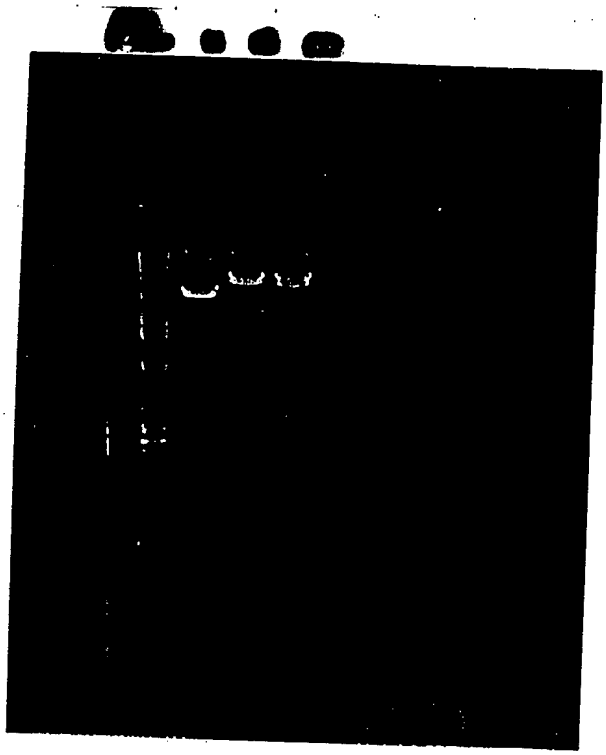


The human RNA doesn't look so great on this gel.

KODAK SAFETY FILM 4RCI

KODAK SAFETY FILM

Photo :



11 - no insert

12 - wrong size

20 - probably cat rotation

10 mini preps of PCR products - putative Hu Cortistatin fragments of 200 + 250 bp. To ppt, rinse + resuspend in 100 µl T.E. Cloned in pCR™ 2.1 vector - Invitrogen. See next page for map.

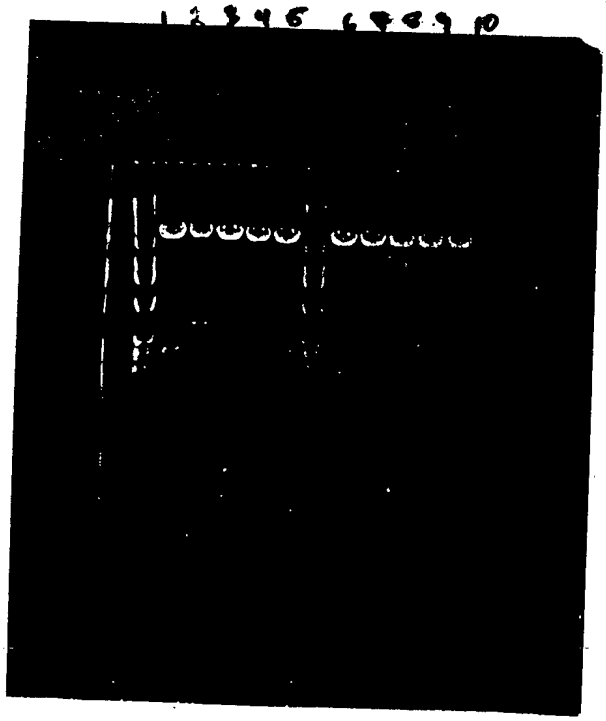
Aliquot 5 µl each of above mini preps. Make a mix for EcoRI digests: 11X

58.3 µl dH_2O
13.2 µl 10X H buffer
5.5 µl EcoRI
Add 7 µl to each tube

Digest 1¼ hrs. @ 37°C. Add 3 µl loading buffer/dye.

Run @ 55 volts on FMC Seakem LE 2% agarose (24 well format) gel in 1X TBE w/ EtBr.

Photo:



Spin down Hu 1, 2, 4 plasmid preps + Rat 11 & 12
Resuspend in T.E. + make 1:100 dil for O.D.

	Vol	Sample	260	280	260/280	Conc	Total
re-picks of 11 + 12	100	R-11	0.288	0.154	1.9	1.44	144 µg
thrown out after gel	100	R-12	0.182	0.098	1.9	0.91	91 µg
	250	Hu 1	0.572	0.301	1.9	2.86	[redacted] 715 µg
	250	Hu 2	0.324	0.171	1.9	1.62	405 µg
	250	Hu 4	0.557	0.290	1.9	2.79	696 µg

Cut 10 µg Hu 4 for making probe to screen Human Library:

~~1.5~~ µl DNA
~~32.5~~ µl dH_2O
4 µl 10 x H buffer
~~2~~ µl EcoRI
40 µl

Add 10 µl dye
2 lanes

Run on LMP agarose gel w/ Marker
See next page for photo.

Photo of LMP agarose gel:

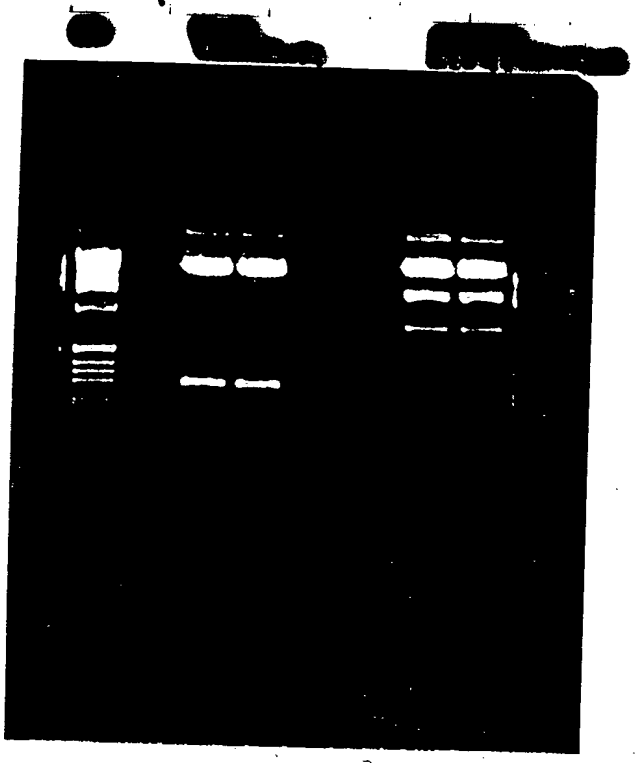
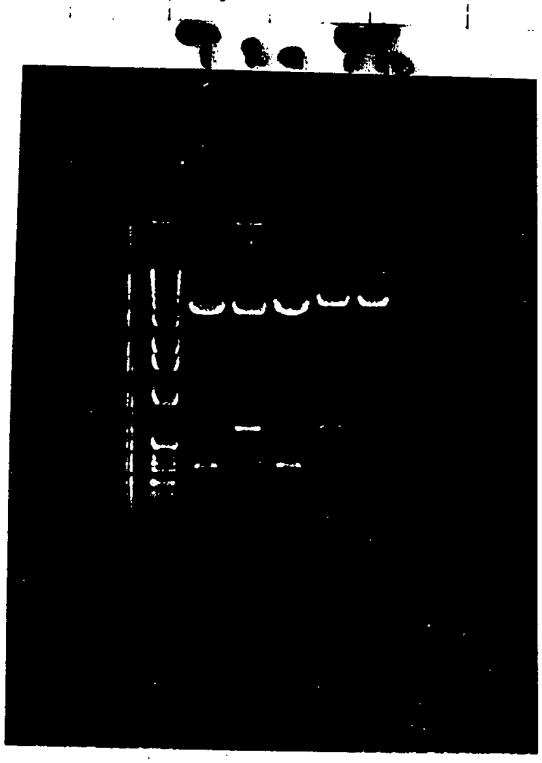


Photo of FMC gel:



Tare = 1.36

Hu4 = 1.45 - 1.36 = 90 µl

$$\frac{\frac{250}{4150} \times 10}{90} = \frac{602}{90} = 6.7 \text{ ng/µl}$$

Use 7 µl for ~50 ng labeling aliquot.

Hu 1, 2, 4 = 0.5 µl DNA
Rat 11, 12 = 1.0 µl DNA
17.0 or 16.5 µl dH_2O
2.0 µl ~~BamHI~~ or ~~EcoRI~~
0.5 µl BamHI or EcoRI
20 µl

NB: Rat 11 is probably contestation – 12 is the wrong size!

→ Label 6 x 50 ng @ 37°C for 30', 0/N @ R.T.

Labeling:

7 µl	DNA } x 6
25 µl	dH_2O }
1 µl	BSA (10 mg/ml)
10 µl	5XOLB
5 µl	α^{32}P dCTP (3000 C:/mMol)
2 µl	Klenow (2U/µl)
50 µl	

Add to each tube

boil 8' → ~42°C

30' @ 37°C, O/N @ RT.

Heat probes @ 68°C for 10'.
Add 50 µl TE-OH ⇔ for 1XTNE + pre warmed.
Vortex. Spin.
Put aqueous phase over prepared G-50 probe quant Columns.

Count 2 µl of each labeled probe:

USER: 3 ID:32P
PRESET TIME: 1.00
PRINTER:EDITED
RS232: OFF

COMMENTS:32P
H#: NO
SCR:YES
RCM:YES
SAMPLE REPEATS: 1
REPLICATES: 1
MULTIPLIER: 1.000000

DATA CALC: C
COUNT BLANK: N
VIAL SIZE: MAX

ISOTOPE 1: 32P %ERROR: 0.50 BKG. SUB: 0 HALF LIFE:YES

SAM NO	POS	TIME MIN	SCR	32P CPM	32P %ERROR	RCM	ELAPSED TIME
1	1-1	1.00	0.889	45.00	29.81	1.24	1.50
2	1-2	0.10	1.000	2922037.8	0.37	0.01	2.91 - Hu 41
3	1-3	0.10	1.000	2983095.5	0.37	0.01	4.34 - 2
4	1-4	0.10	1.000	3035919.0	0.36	0.02	5.78 - 3
5	1-5	0.05	1.000	3593257.8	0.47	0.01	6.84 - 4
6	1-6	0.05	1.000	3280587.3	0.49	0.02	7.88 - 5
7	1-7	0.10	1.000	2746072.5	0.38	0.02	9.22 - 0

Sequencing shows that Hu 1, 4, 5 are the correct fragment of Human contribution.

Pam "zapped" the human brain library in pT7T3D - Eco - NoT into sme cells & checked # of transformants on plates - She used 15 ng!.

10^{-1} dilution, 1 µl = 4000 colonies

Lurs spread plates @ ~ 6:30 pm & put in incubator - 25 plates

Removed plates from incubator @ 9:45 am. Lifted onto Bio-trans filters. Denatured, neutralized, air dry, crosslink, prewash & put filters into prehyb @ 12:30 pm. Std. Southern PH/H solution - 200 ml. Plates returned to incubator to "grow-out" @ ~ 11 am. Remove to RT & then cold room at _____ pm.

Lurs will add 4X probe (Hu 4) @ 7-8 pm tonight.

Wash library screening filters to 0.2 x SSC @ 68°C & put on film w/ 2 screens O/N.

Develop films.
Results: 2 positives! - plate 8 + plate 24
Picked to 1ml LBAmp culture in afternoon.

Luis spread serial dilutions on small + large plates - he also lifted them, reagrew the plates + probed the filters + washed on.
Probe used was 40 ml of the 200 ml (PH/H +3 batches of probe) from primary screen of last week.

Develop secondary/tertiary (skipped grid step since there were only 2 putative positives!) screen film.

Start O/N cultures 3X 100 ml each of Hu 8 + Hu 24. Labeled: HuCort 8 & HuCort 24 + put away 1ml glycerol stock. (Picked 1 positive colony for 8 + 24 into 3 ml LBAmp @ 3pm. Grow until 5 pm. Put 700 µl into 300 µl glycerol + put 0.5 ml to each 100 ml LBAmp in 500 ml flasks + grow O/N.

Std Qiagen tip 500 preps of 100 ml O/N cultures. To ppt w/ isopropanol + then NH_4OAc + EtOH. Resuspend combining 3X 8 + 3X 24 in 1 ml each: 1:100 dil for O.D.

	260	280	260/280	Conc	Total
HuCort 8	0.329	0.176	1.9	1.645	1,645 µg
HuCort 24	0.241	0.130	1.9	1.205	1,205 µg

Aliquot 4 x 10 µg of each for sequencing

Aliquot 2 x 1 µl of HuCart 8 + HuCart 24 for EcoRI - NotI digest:

1 µl DNA
8.8 µl dH_2O
1.2 µl 10 x H buffer
0.5 µl EcoRI
0.5 µl NotI
12 µl

yes! #8 is contamination!

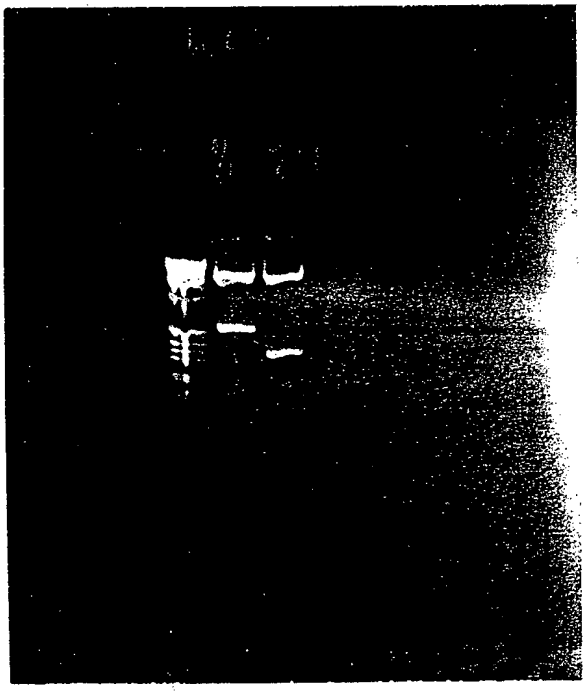


EXHIBIT B

Dr. J. Gregor Sutcliffe
Chairman
Scientific Advisory Board

DIGITAL GENE TECHNOLOGIES, INC.
Total Gene Expression

RECEIVED

H.E.W.M.-P.

William Schmonsees, Esq.
Heller Ehrman White & McAuliffe
525 University Avenue
Palo Alto, California 94301-1900

Dear Bill:

Enclosed are a draft of the cortistation manuscript and a diskette with the sequence figures and the manuscript.

Greg

J. Gregor Sutcliffe, Ph.D.

Enclosures

11149 North Torrey Pines Road, Suite 110 • La Jolla, California 92037 • Telephone: (619) 552-1400 Fax: (619) 552-8625

Cloning, mRNA expression and chromosomal mapping of mouse and human preprocortistatin

Luis de Lecea[1], Pilar Ruiz-Lozano [2], Patria E. Danielson [1],
Jessica Peelle-Kirley [3], Pamela E. Foye[1], Wayne N. Frankel [3], J. Gregor Sutcliffe [1]

[1] Department of Molecular Biology
The Scripps Research Institute
La Jolla, CA 92037

[2] Department of Medicine
University of California, San Diego
San Diego, CA 92093

[3]The Jackson Laboratories
Bar Harbor, ME 04609

Abstract

Cortistatin is a 14 residue putative neuropeptide with strong structural similarity to somatostatin and is expressed predominantly in cortical GABAergic interneurons of rats. Administration of cortistatin into the brain ventricles specifically enhances slow wave sleep, presumably by antagonizing the effects of acetylcholine on cortical excitability. Here we report the cDNA cloning of the mRNAs encoding mouse and human preprocortistatin and the mRNA distribution and gene mapping of mouse cortistatin. Analysis of the nucleotide and predicted amino acid sequences from rat and mouse reveals that the 14 C-terminal residues of preprocortistatin, which is the sequence that is most similar to somatostatin, are conserved between species. Lack of conservation of other dibasic amino acid residues whose cleavage by prohormone convertases would give rise to additional peptides suggests that cortistatin14 is the only active peptide derived from the precursor. As in the rat, mouse preprocortistatin mRNA is present in GABAergic interneurons in the cerebral cortex and hippocampus. The preprocortistatin gene maps to mouse Chromosome 4, in a region showing conserved synteny with human 1p36. The human putative cortistatin peptide has an arginine for lysine substitution compared to the rat and mouse products, and is N-terminally extended by three amino acids.

Introduction

We recently isolated a cDNA clone of the mRNA encoding rat preprocortistatin, a 112-residue protein whose amino acid sequence suggests that is the putative precursor of a novel secreted neuropeptide (1). Maturation of the rat preprospecies to procortistatin would produce a protein that could be processed at either an Arg-Arg site to generate a 29-residue peptide (rCST29), at a Lys-Lys site to give rise to a 14 amino acid peptide (rCST14), also called cortistatin, or at both sites to generate both CST14 and a 13-residue peptide (Fig 1). rCST14 shares 11 of its 14 residues with somatostatin, including those that are known to be responsible for somatostatin binding to its receptors (2) and the cysteines that are likely to render the peptide cyclic. The 13-residue species is unrelated to known proteins. Preprocortistatin mRNA is expressed in a distinct subset of interneurons in the rat cerebral cortex and hippocampus, areas of the brain thought to be important for high cognitive functions (1). The cDNA sequences of preprocortistatin and preprosomatostatin indicate clearly that they are the products of separate genes.

Synthetic rCST14 was shown to share several biological properties with somatostatin: it bound to somatostatin receptors on GH_4 pituitary cells, inhibited the VIP- and TRH-induced accumulation of cAMP in those cells, and depressed neuronal activity in hippocampal neurons, probably by enhancing the potassium M-current (1). However, the effects of cortistatin on cortical electrical activity and sleep were distinct from those found for

somatostatin. Intracerebroventricular administration of synthetic rCST14 specifically enhanced the amount of time that the animals spent in slow wave sleep but did not affect significantly their paradoxical (REM) sleep. Moreover, rCST14 was shown to antagonize the effects of acetylcholine on cortical measures of excitability, whereas somatostatin is known to enhance acetylcholine release and potentiate acetylcholine responses (1). These observations demonstrated that cortistatin is functionally distinct from somatostatin and raised the possibility that cortistatin exerts its activities through an uncharacterized cortistatin-selective receptor, although other explanations of different functionalities can be considered.

To gain information on the conservation of the putative processed neuropeptides we have isolated cDNA clones encoding mouse and human preprocortistatin. We demonstrate that the amino acid sequence of the active cortistatin-14 peptide is fully conserved in mouse. Lack of sequence conservation for the 13-residue peptide suggests that it may not be an active proteolytic product of preprocortistatin. In addition, we have mapped the gene to mouse chromosome 4, in a region syntenic to human chromosome 1p36. The human putative cortistatin peptide has an arginine for lysine substitution compared to the rat and mouse products, and is N-terminally extended by three amino acids.

Results

Analysis of mouse preprocortistatin DNA sequence

We previously reported the isolation of a rat cDNA clone whose nucleotide sequence suggested that it encoded the precursor of cortistatin, a peptide with sequence similarity to somatostatin. Preprocortistatin begins with a 27-residue apparent secretion signal sequence. Interestingly, this region contains six iterations of the trinucleotide CTG, whose triplet expansion in other genes has been implicated as causal in neurological diseases (e.g. myotonic dystrophy) (3). The rat preprocortistatin deduced amino acid sequence contains several pairs of basic residues that are possible substrates of prohormone convertases. Cleavage at all basic amino acids pairs would give rise to rCST17 (with a putative amidation site), rCST31, rCST29, rCST13 and rCST14 (Fig. 1). Alternative or partial cleavage could produce additional peptide products. rCST14 may be further processed by carboxypeptidases that would remove its C-terminal lysine.

We used the full- length rat cDNA clone to screen a mouse cerebral cortex cDNA library (generously provided by Dr. K. Hasel). Several positive clones were isolated and their nucleotide sequences determined. Two cDNA clones, 430 bp long, appeared to be full-length as judged by the alignment of their 5' ends with the rat sequence (not shown). After introduction of two gaps, the mouse and rat nucleotide sequences were 86% identical (Fig 2A). Assuming that the putative mouse translation initiation product begins at the second methionine triplet, it contains 108 amino acids compared to 112 for rat. Again, after introduction of two gaps, the putative rat and mouse proteins share 82% identity (Fig 2B). The mouse nucleotide sequence corresponding to rCST14 and the

adjacent lysine doublet that putatively serves as its site of proteolytic release from its precursor were identical to same region in the rat sequence, thus supporting a functional conservation of the mature peptide. The DNA sequence upstream from the processing site of mCST14 showed several points of divergence, including some resulting in non-conservative amino acid substitutions. Two of these differences obliterate pairs of basic residues (Fig 1, 2B), suggesting that CST14 is the only active peptide processed from both the rat and mouse precursors.

Cloning of human preprocortistatin

To isolate a DNA clone encoding human preprocortistatin we used a combination of PCR and conventional screening techniques. We isolated a 120 bp fragment of the human coding sequence by PCR using degenerate primers from the mouse and rat sequences. The nucleotide sequence of this fragment was compared to the EST database and one sequence was found with significant similarity to cortistatin, although several gaps were required for alignment. We then designed primers to amplify a 250 nucleotide fragment that was used as a probe to screen a human whole brain cDNA library. From several rounds of screening we isolated two cDNA clones, 450 and 270 nucleotides in length and the sequence from the longest was determined.

The human nucleotide sequence (Fig 2A) showed a much lower degree of identity to the rat sequence (71%). The human preprocortistatin deduced amino acid sequence (Fig 2B) has 114 residues and begins with a 29- amino acid hydrophobic probable secretory

signal sequence. The sequence corresponding to the putative signal peptide of preprocortistatin contains only four iterations of CTG encoding the amino acid leucine, in contrast to six iterations of the same triplet in the rat peptide precursor or three in mouse, suggesting that this sequence is unstable and subject to expansion. Analysis of the putative processing sites in human preprocortistatin revealed that it may be cleaved at two RR sites, giving rise to hCST29 and a C-terminal seventeen residue peptide that shared 13 of the last 14 residues with rat and mouse CST14, here called hCST17. The Lys-Lys pair that lies just N-terminal to cortistatin-14 in rat and mouse is not conserved in the human sequence. The other possible products that follow the signal sequence (hCST21 and hCST31) are not very conserved across species, although rCST31 and hCST31 share 13 residues clustered in their N-terminal regions that are conserved among the rat, mouse and human prohormone sequences (Fig 2B).

mRNA expression

We determined the distribution of preprocortistatin mRNA by Northern blot. A band of approximately 600 nucleotides was detected in samples prepared from rat brain, cortex and hippocampus, but not pancreas or gut (Fig 3) or adrenal gland, liver, spleen, thymus, ovary, testes, anterior pituitary (not shown). This pattern of expression is clearly distinct from somatostatin mRNA, which is present in several endocrine tissues. Hybridization to northern blots of mouse tissues revealed the presence of two bands in brain but not liver, kidney or thymus. Two bands were also observed in the human brain sample. These bands are probably due to alternate polyadenylation signals, found to be present in

mouse genomic clones (LdL, unpublished observations) and in human cDNA clones.

We previously reported that rat cortistatin is expressed in a subset of cortical and hippocampal GABAergic interneurons. To determine whether the expression of cortistatin was conserved between species, we performed in situ hybridization with mouse brain tissue (Fig 4). As in the rat, cortistatin positive neurons were enriched in the cerebral cortex and hippocampus. In the temporal/visual cortex, cortistatin positive neurons were especially abundant in layer VI, with very few scattered cells present in layer II-III (Fig 4. A,B). In the hippocampus, preprocortistatin mRNA could be visualized in the stratum oriens of the CA1-CA3 fields, as well as in a few neurons adjacent to the granule cell layer of the dentate gyrus. The hilar region was totally devoid of preprocortistatin expressing cells. Strong signals could also be detected in the amygdala, especially in the medial amygdaloid nucleus (Fig.4C). In the hypothalamus, preprocortistatin mRNA was detected in a few cells in the periventricular nucleus.

Chromosomal mapping of mouse cortistatin

We mapped the cortistatin gene (gene symbol, *Cort*) by single-strand conformation polymorphism (SSCP) analysis of a panel of interspecific backcross mouse DNAs. We designed primers that spanned the 3' coding/3' untranslated sequence of mouse cortistatin cDNA and amplified the corresponding 107 bp genomic fragment from C57BL/6J (B6) and a strain inbred from wild-derived *Mus spretus* (SPRET/Ei). Representative PCR fragments were sequenced to confirm their identity. A clear

polymorphism was found which distinguished the two strains. To determine linkage the segregation pattern of the B6 allele was followed in subpanel of 54 (B6 x SPRET) F1 X SPRET backcross offspring and compared to that of over 2500 genes previously mapped on the panel. The mouse cortistatin locus was found to lie on distal Chromosome 4 - in contrast with the somatostatin gene which maps to Chromosome 16 (4) - and was non-recombinant with the *Mtfhr* locus (LOD 16.3; Figure 5). Neurological mutations that are known to reside in this region include Wallerian degeneration (*Wld*) and jerker (*je*). A quantitative trait locus for beta-carboline induced seizures has also been mapped in this region (5). This telomeric region of mouse Chr 4 show strong conserved synteny with human chromosome 1p36 (6), but we have not identified human neurological disorders mapping to this region for which *Cort* would be a compelling candidate.

Discussion

We have described the cloning of the mouse and human homologues of the neuropeptide cortistatin mRNAs and mRNA distribution and gene mapping of mouse preprocortistatin. Analysis of the nucleotide and predicted amino acid sequences from rat and mouse reveals that the 14 C-terminal residues of preprocortistatin, which is the sequence that is most similar to somatostatin, are conserved between these species, whereas the mono or dibasic amino acid residues whose cleavage by prohormone convertases would give rise to additional peptides are not conserved.

From the known members of the family of precursor convertases only furin, PC1 and

PC2 are expressed at significant levels in neurons (7). Furin normally cleaves precursors entering the constitutive pathway and has strong substrate specificity. In general, the available cleavage specificity data demonstrate that both PC1 and PC2 prohormone convertases cleave precursors at single and pairs of basic residues and that the four combinations KR RR, RK and KK are possible cleavage sites for these enzymes (7). In the mouse preprocortistatin cDNA sequence, the only processing site that is conserved with rat is the one that gives rise to CST14. Interestingly, the arginine from the KR site that would produce CST29 in rat, is substituted by a serine in mouse, generating a KS sequence, a very unlikely substrate for convertases. Even though the KK site is not a preferred substrate for PC1 or PC2, there are examples in the literature of such cleavage, especially in cells of neural origin. For example, PC1 has been shown to cleave human proenkephalin at a Lys-Lys site (8). Also, β-endorphin can be efficiently cleaved at its Lys_{28}-Lys_{29} site in arcuate hypothalamic neurons, generating a potent endogeous opioid antagonist (9). Furthermore, the KK site in the N-terminus of beta-melanin stimulating hormone (β-MSH) can be generated from proopiomelanocortin (POMC) by PC2 cleavage in the intermediate lobe of the pituitary (10).

Analysis of human preprocortistatin processing sites shows relative conservation with the rat sites and, noteworthy, the presence of a RR site that would give rise to a 17-residue peptide that contains the active cortistatin 14 sequence with one conservative substitution. This suggests that the critical amino acids for cortistatin function reside in the loop formed by the two cysteines and possibly, in the N-terminal proline and C-

terminal lysine, although the latter may be processed by carboxypeptidases in the secretory pathway (11). We cannot rule out the possibility that the human CST species is further processed at a single R site, to generate CST14 with an additional N-terminal methionine substitution.

Recently, a second vertebrate somatostatin gene has been reported in the frog *Rana ridibunda* (12). Frog somatostatin II has two amino acid substitutions relative to somatostatin I: a Pro at position 2 and a Met in position 13. Thus, the N-terminal proline residue may be critical for the specific actions of somatostatin II in frog and cortistatin in rat, mouse and human. However, somatostatin II is an unlikely predecessor of cortistatin, as the nucleotide and amino acid sequences of the precursors are quite divergent. As during the evolution of tetrapods several gene duplications may have occurred, the existence of more members of the somatostatin/cortistatin family in mammals cannot be ruled out.

Analysis of mouse preprocortistatin mRNA expression showed an overall coincidence with the pattern described in rat. However, mouse preprocortistatin mRNA seems less abundant than its rat counterpart, as judged by northern blot and in situ hybridization. In the mouse visual cortex, cortistatin-positive cells were abundant only in the deep layers whereas in rat, cortistatin signals covered the entire thickness of the cortex. Also, we could detect some cortistatin-positive cells in the mouse periventricular hypothalamic area and in the amygdala, regions that were negative in the rat. Small differences in the

expression of neuropeptides between species have been reported for galanin and other neuropeptides (13, 14), although the functional implications are unknown.

The rat DNA sequence for preprocortistatin contains six repetitions of the trinucleotide CTG in the region corresponding to its putative signal peptide, wherease the mouse sequence contains three and the human displays four iterations of the same triplet. The instability of CXG repeats has been shown to be responsible for several neurological diseases in humans as well as in mouse models. Expansion of the CTG repeat of cortistatin would likely impair its processing into a mature, active peptide. Alternatively, an expanded poly-leucine stretch could produce gain-of-function mutations.

Materials and Methods

DNA cloning and sequencing

A mouse (C57BL/6J) cortex DNA library (kindly provided by K. Hasel) was screened with the full-length rat cortistatin DNA. Replica filters containing 1.5x 10^5 colonies (30 plates of 5000 each) were washed at moderate stringency (1xSSC 68° C). After several rounds of screening we isolated five positive clones and the sequence of the longest was determined by the dideoxy chain termination method. Human preprocortistatin cDNA was amplified by PCR using primers to the C-terminal sequence of cortistatin. The PCR fragment was cloned, random prime labeled and used to screen a cDNA library prepared from human whole brain mRNA (Clontech).

Northern blot

Cytoplasmic poly A + RNA was isolated from rat and mouse brains as described (15). Two micrograms of polyA + RNA from rat, mouse and human (Clontech) samples were run on formaldehyde agarose gels and transferred to nylon filters as described (16). Mouse or human cDNA probes were labeled with ^{32}P and random primers.

In situ hybridization.

C57BL/6J mice were perfused with 4% paraformaldehyde and processed for in situ hybridization as described (17). Free floating sections were hybridized with 10^7 cpm/ml of labeled cortistatin probe, and washed at 60°C in 0.5xSSC/50% formamide for 3 h. After mounting, slides were dipped in Ilford K5 emulsion diluted in water, and exposed for 3 weeks at 4°C. Slides were developed in Kodak D19, counterstained, and mounted in Permount.

Chromosomal mapping.

The oligonucleotides for mapping *Cort* by SSCP were : 5'-AAAAAGCCCTGCAAGAACTT-3'; and 5'-ATTCAGGTCTCGTTGGCATC-3'. The PCR conditions have been described previously (18) except that α^{32}P-dCTP was incorporated into the reaction. PCR product was denatured, then quick cooled on ice and electrophoresed for 4 hr in a 0.5% MDE gel (AT Biochem, Inc) at 4°C. The gel was exposed to X-ray film overnight. Linkage data were analyzed using latest version of the computer program MapManager (19) which can be obtained on the web

(http://mcbio.med.buffalo.edu/mapmgr.html). The sequence of the *Cort* PCR product was determined by the dideoxy method using Cort1F and Cort1R primers.

Acknowledgments

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Figure legends

Figure 1. Schematic drawing (not to scale) showing the structure of the rat, mouse and

human cDNAs encoding preprocortistatin and putatively processed fragments. The putative cleavage sites by prohormone convertases have been indicated (RR, KR, KK and RK). Putative products are labeled by species (r, m, h) and predicted amino acid length in the absence of further processing (e.g. rCST14).

Figure 2. A. Alignment of the nucleotide sequences of rat, mouse and human preprocortistatin cDNAs. The human sequence is a composite from different PCR fragments and cDNA clones, including one that showed a deletion in the coding sequence and an additional 3' polyadenylation signal. The CTG repeat that encodes the amino acid leucine, and that is of variable length between species has been underlined. The two possible polyadenylation signals are marked with an asterisk. Nucleotides conserved among all three species are shown uppercase; otherwise, lowercase. B. Alignment of the deduced amino acid sequences of the rat, mouse and human cortistatin precursors. The putative dibasic cleavage sites are indicated in bold. Consensus residues are indicated. C. Comparison of the amino acid sequences and predicted secondary structures of rat, mouse and human cortistatin and somatostatin from frog and mammals.

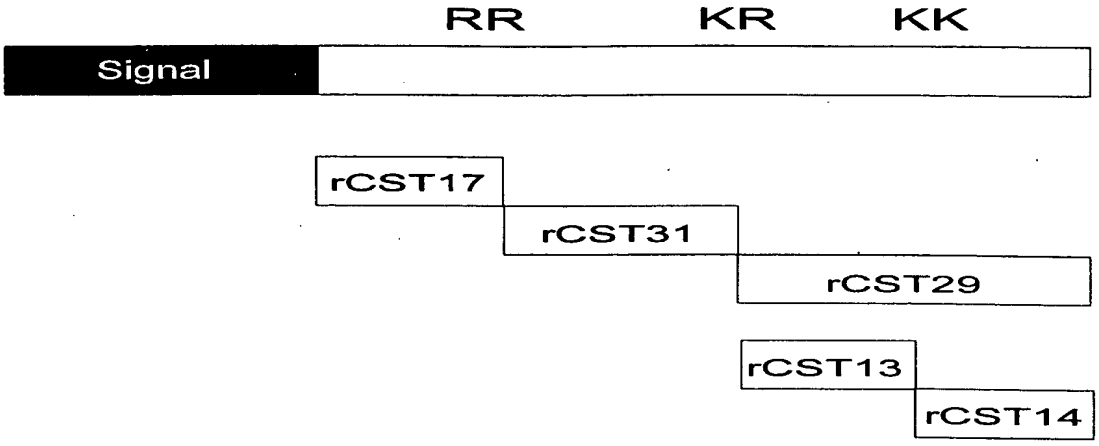
Figure 3. Northern blots of RNA samples from rat whole brain, cortex, hippocampus, gut, pancreas, mouse brain, liver, kidney. The blots were hybridized with the rat cortistatin cDNA and with a cyclophilin probe (20; not shown) as a control for loading and RNA integrity. A separate northern blot containing an mRNA sample from whole human brain was hybridized with the human preprocortistatin cDNA sequence. In short

exposures both the mouse and human samples displayed two bands, pobably generated by alternative polyadenylation signals.

Figure 4. In situ hybridization in mouse brain. A.- Dark field micrograph of a section through the mouse cortex. Note the presence of scattered cells in the deep layers of the neocortex and hippocampal CA1 field (arrows). B.- High magnification of a cortistatin positive cell in layer VI. C. Dark field image of the mouse amygdala hybridized with a cortistatin riboprobe. The amygdala and several regions of the hypothalamus (not shown) showed stronger signals in the mouse compared to the rat.

Figure 5. Chromosomal mapping of mouse cortistatin. Genetic map of the mid-distal portion of mouse Chromosome 4 showing selected markers typed on the interspecific backcross on the right, and map distances between them, in cM, on the left. The marker D4Bir1 is the nearest published marker on this cross to the Chr 4 telomere. Genotype data and citations for these markers can be found on The Jackson Laboratory WWW home page http://www.jax.org.

Rat



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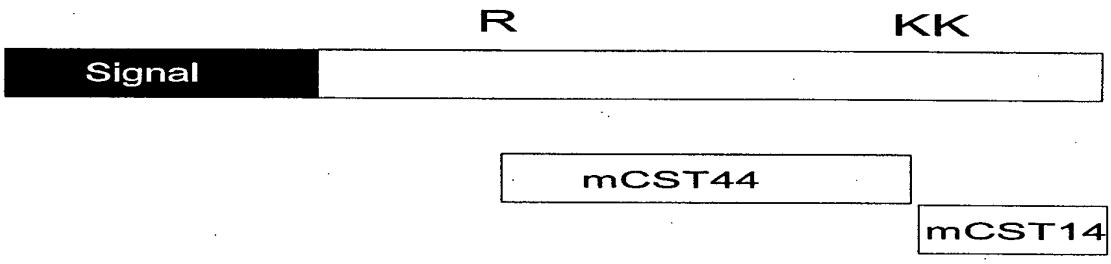

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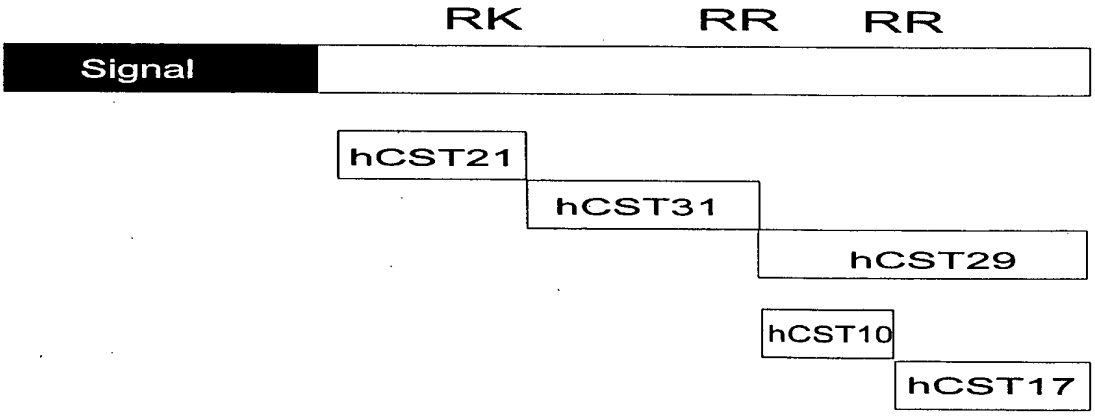

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Rat ct       .......... .aaagcacag acTtcaggtc tCCaaGgAgG AtGgGtgGCt
Human cst    gcacgaggcc aaaacattga ttTcagggct gCCagGaAgG AaGaGcaGCa

Mouse cst    GagGcacagG AGgcAAGtgg CccTCAG... .......... .....CCttC
Rat cst      GcaGcacaaG AGgcAAGcgg CcgTCAG... .......... .....CCctC
Human cst    GcaGggtggG AGagAAGctc CagTCAGccc acaagatgcc attgtCCccC

Mouse cst    gG........ .GCTGCTGCT gctCtgGGgg gtcGCagCCt CcGCCCTtCC
Rat cst      aGtctgctgc tGCTGCTGCT gctCtcGGgg atcGCagCCt CtGCCCTcCC
Human cst    gGcctcctgc tGCTGCTGCT ctcCggGGcc acgGCcaCCg CtGCCCTgCC

Mouse cst    CCTGGAGaGt GGcCCtACtG GCCagGACAG ......TgTG CAGGAaGCca
Rat cst      CCTGGAGaGc GGtCCcACcG GCCagGACAG ......TgTG CAGGAtGCca
Human cst    CCTGGAGgGt GGcCCcACcG GCCgaGACAG cgagcaTaTG CAGGAaGCgg

Mouse cst    C...cgaggG GAggAgCgGC CTtCTGACTT TCCTtGCcTG GTGGcacGAG
Rat cst      CaggcgggaG GAggAcCgGC CTtCTGACTT TCCTtGCcTG GTGGcatGAG
Human cst    CaggaataaG GAaaAgCaGC CTcCTGACTT TCCTcGCtTG GTGGtttGAG

MOUSE CST    TGGgCtTCCC AaGcCAGctC CaGcaCCccc gTcGgAGgGG gtaCCCccGg
Rat cst      TGGgCtTCCC AaGaCAGctC CaGcaCCgct tTcGaAGgGG gtaCCCcgGa
Human cst    TGGaCcTCCC AgGcCAGtgC CgGgcCCctc aTaGgAGaGG aagCCCggGa

Mouse cst    GcTGtCcAaG aGcCAGGAAa GgcCACCCCc CCAaCAgcCC cCaCaCCtGG
Rat cst      GcTGtCtAaG cGgCAGGAAa GacCACCCCt CCAgCAgcCC cCaCaCCgGG
Human cst    GgTGgCcAgG cGgCAGGAAg GcgCACCCCc CCAgCAatCC gCgCgCCgGG

MOUSE CST    AtAaAAaGCC CTGCAaGAAC TTCTTCTGGA AaACCTTCTC CTCgTGCAAg
Rat cst      AtAaAAaGCC CTGCAaGAAC TTCTTCTGGA AaACCTTCTC CTCgTGCAAg
Human cst    AcAgAAtGCC CTGCAgGAAC TTCTTCTGGA AgACCTTCTC CTCcTGCAAa

Mouse cst    TAaccCcacc CtgggcataG Caccctggcc acCctgtgag atgccaacga
Rat cst      TAgccCgagc CtgaccggaG Cctgaccggc caCcctgtga atgcagccgt
Human cst    TAaaaCctca CccatgaatG C......... .tCacgcaag tgtaatgaca

Mouse cst    GaCCTGAATA AAgacTgTcA Atcaac.... .......... ..........
Rat cst      GgCCTGAATA AAgagTgTcA Agt....... .......... ..........
Human cst    GaCCTGAATA AAatgTaTtA Agcagcagtg atctttcctc tcctccttcc
                        *
Mouse cst    .......... .......... .......... .......... ..........
Rat cst      .......... .......... .......... .......... ..........
Human cst    caagtcattt gaaaagtgtt tgttatttaa attccaataa tgcccaatac

Mouse cst    .......... .......... .......... .......... ..........
Rat cst      .......... .......... .......... .......... ..........
Human cst    tgacgtgtct tgagtaattt ggaacccaaa gtgaagatct ttgataaaga

Mouse cst    .......... .......... .......... .......... ..........
Rat cst      .......... .......... .......... .......... ..........
Human cst    tttttttttgt ggttcgactg gactgtgctg agtgcgggca ctgggctttt

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Rat cst      .......... .......... .......... .......... ..........
Human cst    cttctgatgt tcattatggt gctgggaagc tctgtctttg atttaaaata
                                                                  *
Mouse cst    .......... .........
Rat cst      .......... .........
Human cst    aaatagctaa aggctacac
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           1                                                        50
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MOUSE CST  MMGGRGTGGK WPSAFGLLLL W...GVAASA LPLESGPTGQ DS..VQEATE
HUMAN CST  .......... MPLSPGLLLL LLSGATATAA LPLEGGPTGR DSEHMQEAAG
Consensus  ---------- -P----LLLL ------A--A LPLE-GPTG- DS---Q-A--

           51                                                      100
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MOUSE CST  G.RSGLLTFL AWWHEWASQA SSSTPVGGGT PGLSKSQERP PPQQPPHLDK
HUMAN CST  IRKSSLLTFL AWWFEWTSQA SAGPLIGEEA REVARRQEGA PPQQSARRDR
Consensus  -----LLTFL AWW-EW-SQ- S---------- ------QE-- P-QQ----D-

           101          116
RAT CST    KPCKNFFWKT FSSCK
MOUSE CST  KPCKNFFWKT FSSCK
HUMAN CST  MPCRNFFWKT FSSCK
Consensus  -PC-NFFWKT FSSCK
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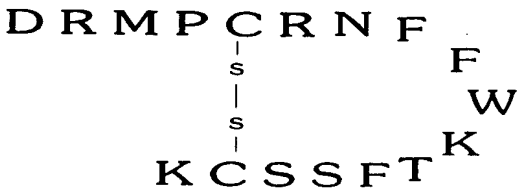
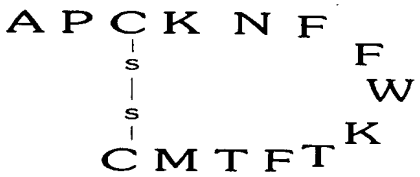


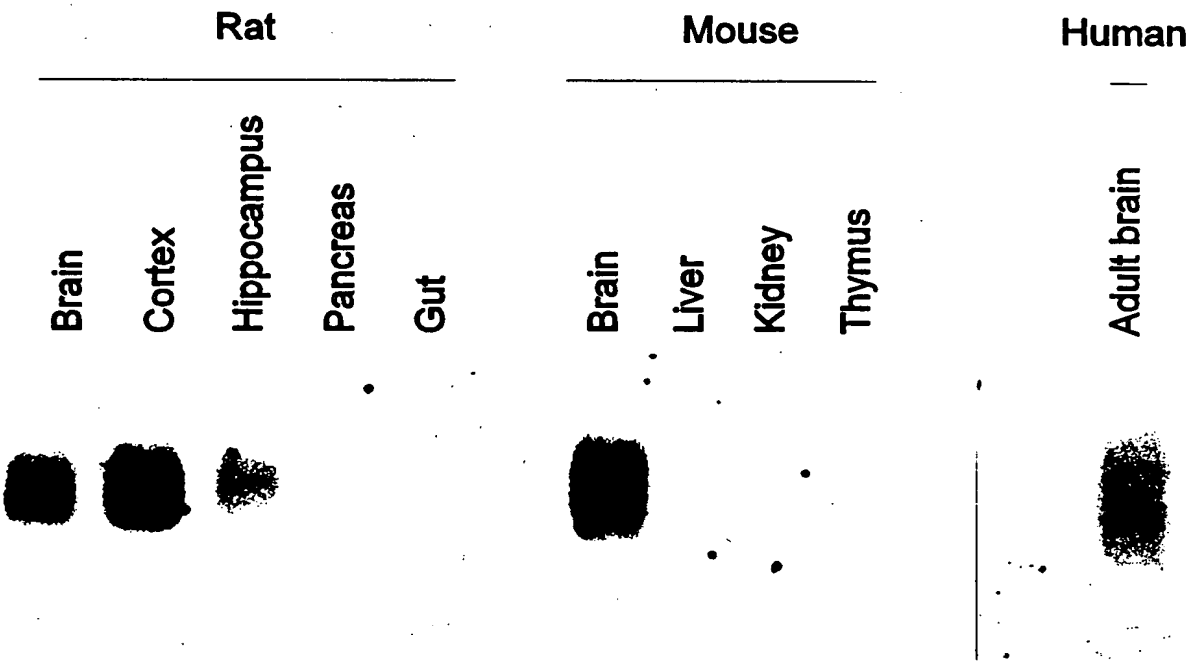
Cortistatin (Human)

Cortistatin (Rat, mouse)



Somatostatin (frog)

Somatostatin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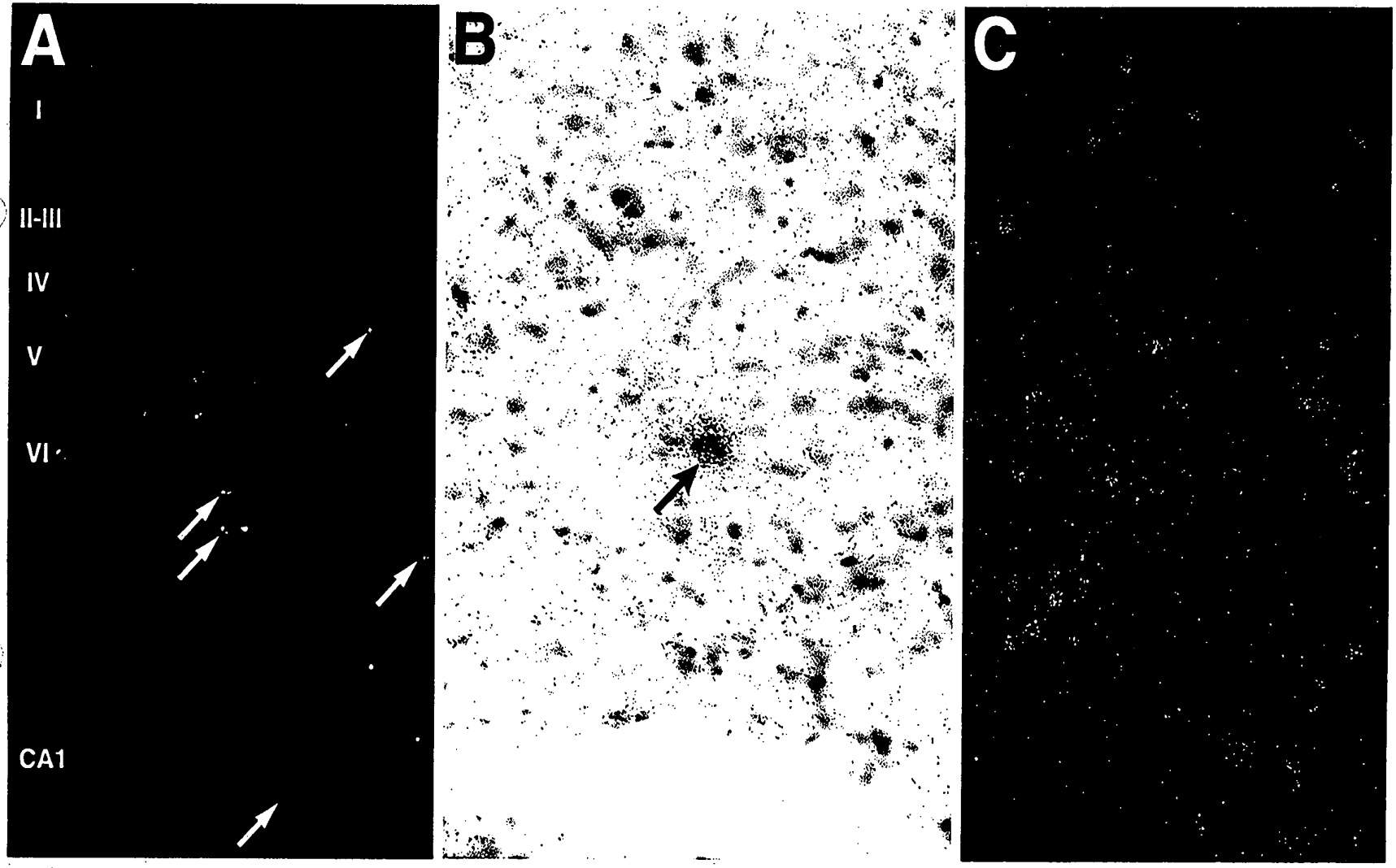
Adult brain


A
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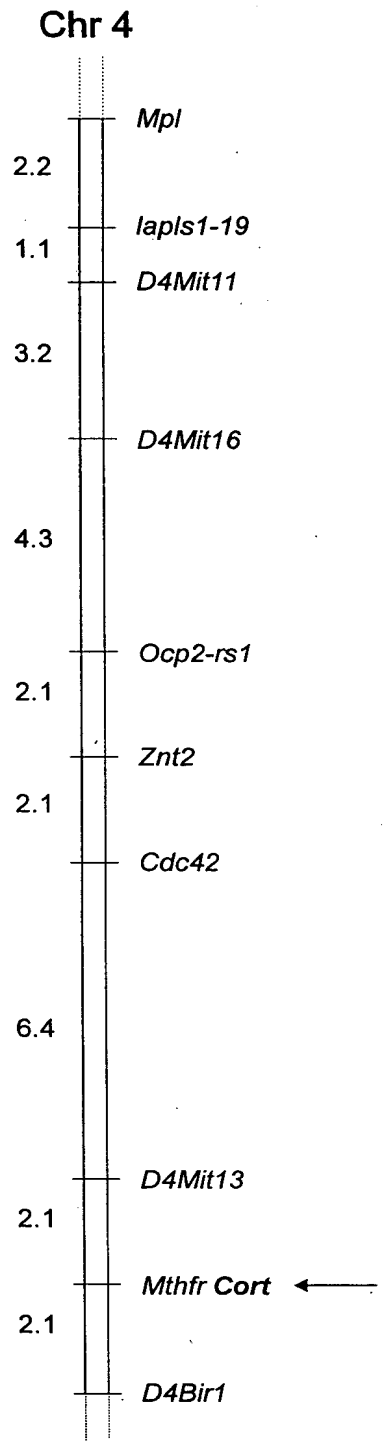
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Znt2
2.1
Cdc42
6.4
D4Mit13
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Mthfr Cort
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D4Bir1

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Corristatin - cloning project

6.3.9(
Revisio
- Pan
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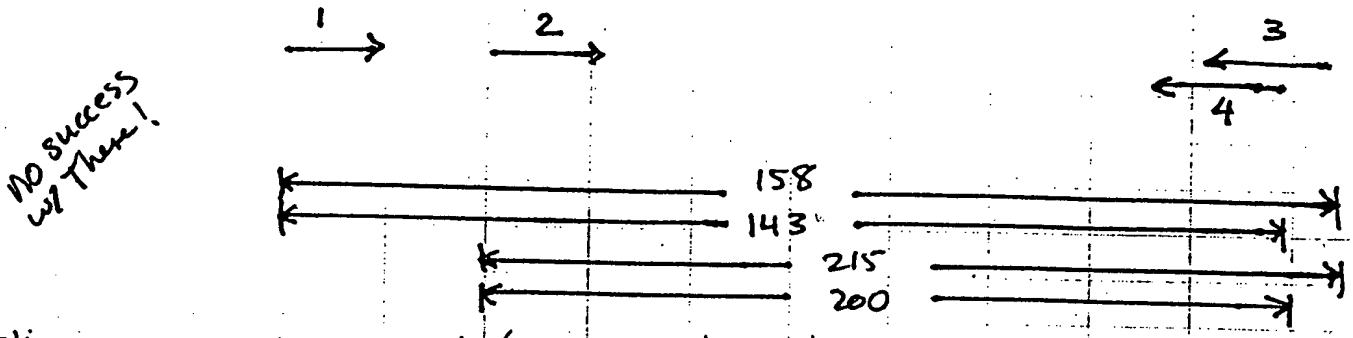


oligos - based on rat/mouse homology:

already made 1) C A A/G G A C/T T C A/C/G/T G T A/C/G/T C A A/G G A

"cst-two" 2) T G G T G G C A T/C G A G/A T G G

"cst-three" 3) C A A/G/C C/G A/T A/G/C C/G T A A/G A A A/G/C G T C/T T T C

"cst-four" 4) T T C C A A/G A A A/G A A A/G T T T/C T T A/G C A

	Tm (ran
17mer	46 -5
15mer	46 -5(
18mer	46 -5(
20mer	46 -5(

PCRs with human brain cDNA library

1. 1+3
2. 1+4
3. 2+3
4. 2+4
5. (1+3) ½ (1+4)
6. (1+3) ½ (2+3)
7. (1+3) ½ (2+4)
8. (2+3) ½ (2+4)
9. (1+4) ½ (2+4)

- P.F. trimmed down same cst-two & cst-three nucleotides- to make all 4 oligos have similar Tm °C
- P.D. double-checked these oligos

10/96

Pam Transformed the mixed miniprep of set estimating positive clones 11, 12 & 20 from 1995. She'll spread plates on 10/16 & grow out O/N.

10/17 I picked 10 single colonies of each, ligation of 11-1 → 11-10, 12-1 → 12-10, & 20-1 → 20-10, and set up 5 ml LB+Amp O/N cultures.

10/18 Put away 100 µl of each prey w/ glycerol @ -70°C.

Alkaline lysis minipreps of 30 clones. Resuspended in 100 µl T.E. & stored @ -20°C O/W E.

10/22 Digest 5 µl of each w/ BamHI

5 µl DNA
5.3 µl dH_2O
1.2 µl 10×B buffer
0.5 µl BamHI

Mix — (35×)
185.5 µl dH_2O
42.0 µl 10×B buffer
17.5 µl BamHI
245 µl
↳ 7 µl per tube.

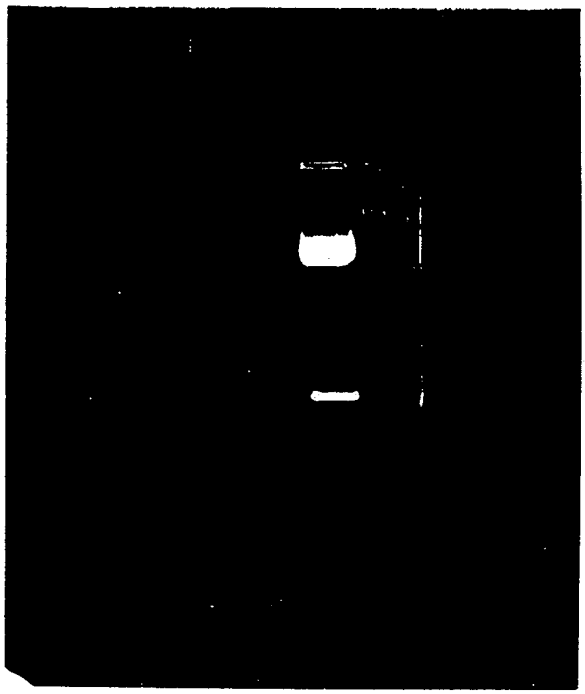
Aliquot 5 µl of each DNA sample & add 7 µl of enzyme digest mix. Incubate 1½ hrs @ 37°C.
Add 3 µl dye

Run on FMC 24 well format Seakem agarose gels in TBE.

NB: no pcr primer.
Lifted filters from spread plates & probed w/ Express Hyb 10/24/96.
Developed film 10/25. (Unexplained +'s (?))
on 10/27 it put in to wash 3×50 ml O/N.

10/21 Cut 5 µg pBSK Cort (0.8 µg/µl) – rat cortisatin ORF probe.

6.25	
14.25	
2.5	
1.0	
1.0	
25 µl	



Run on "old" LMP agarose gel from 9/25/96 – stored wrapped @ 4°C.

Recover insert band.

Tare = 1.38
RatCort ORF = 1.44 − 1.38 = .

(450/3450)5 = 652 ng

652 ng/60 µl ≈ 11 ng/µl

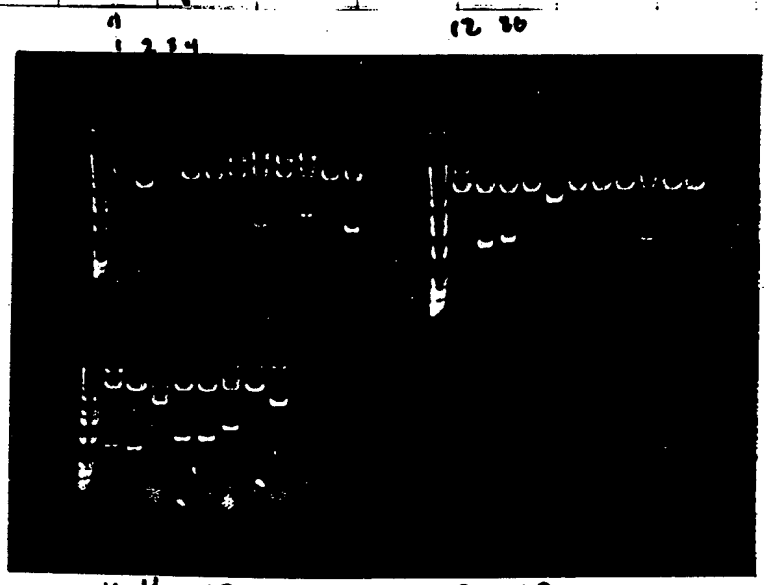
Use 4 µl for labeling

10/22 4 µl DNA
28 µl dH_2O } 100°C for 7' → ~42°C

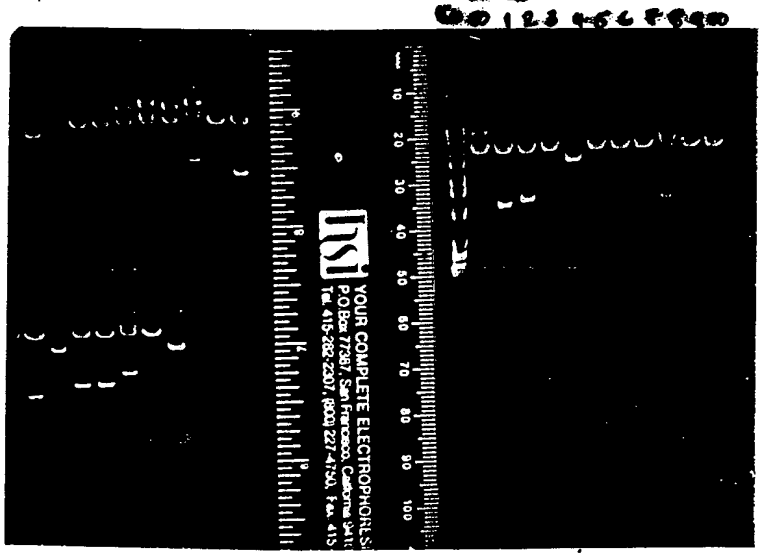
Add 1 µl BSA
10 µl 5X OCB
5 µl α^{32}P dCTP
2 µl Klenow (2U/µl)
50 µl

1 hr. @ 37°C
O/N @ RT.

10/22 Photos of gels:

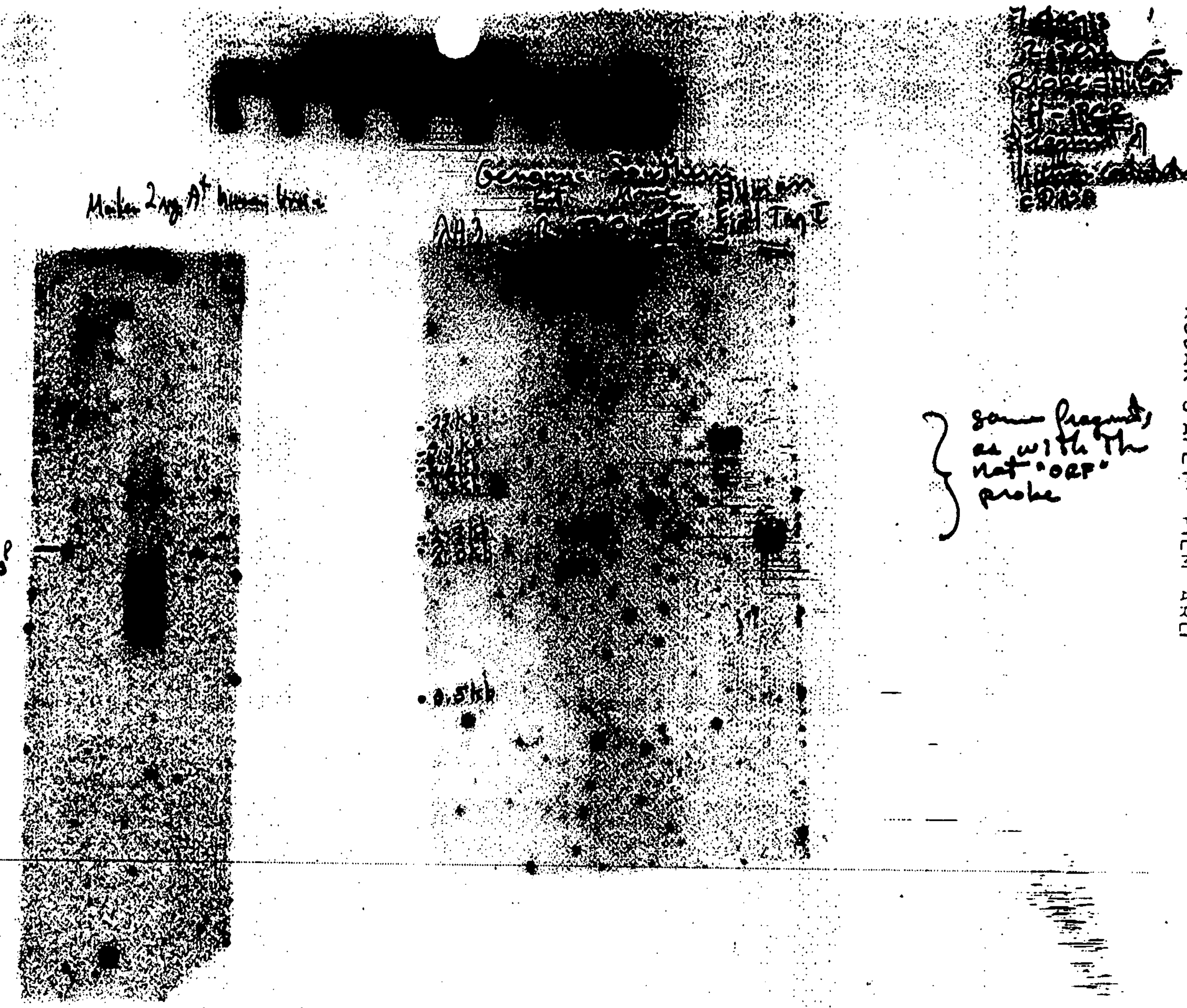


11-4 12-8 20-7 + 20-10



Putative positives:

11-4 (s?)
12-8
20-7
20-10



Marker 2 µg A+ human liver

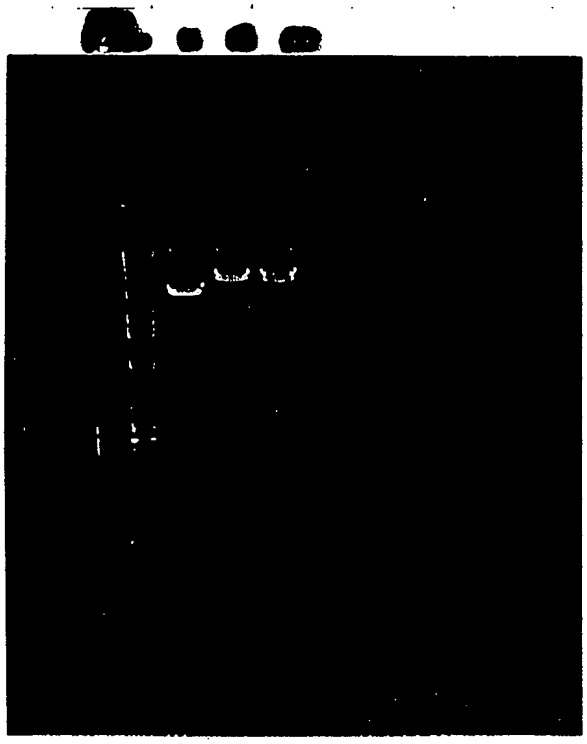
probably cyp runs here!

same fragments as with the rat "ORF" probe

The human RNA doesn't look so great on this gel.

29 October 1996

Photo :



11 - no insert

12 - wrong size

20 - probably contrast

Tuesday 29 October 1996

10 mini preps of PCR products - putative Hu Cortistatin fragments of 200 + 250 bp. To pot, rinse + resuspend in 100 µl T. E. Cloned in pCR™ 2.1 vector - Invitrogen. See next page for map.

Wednesday 30 October 1996

Aliquot 5 µl each of above mini preps. Make a mix for EcoR1 digests: 11X

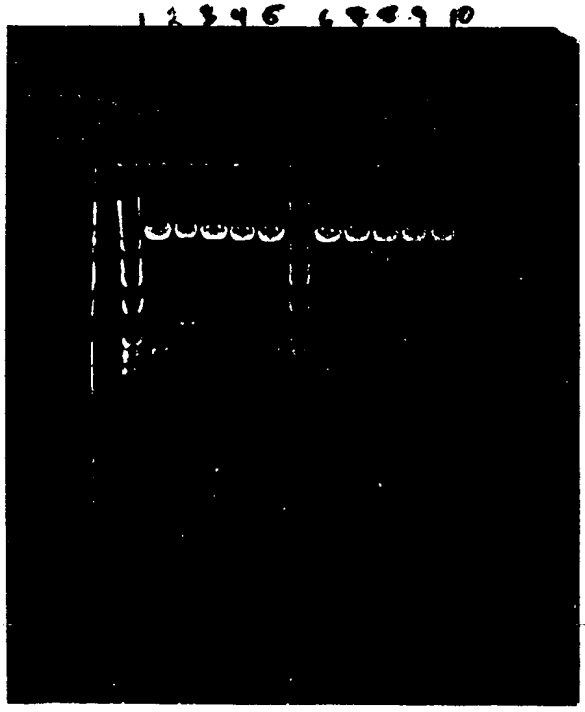
58.3 µl dH_2O
13.2 µl 10X H buffer
5.5 µl EcoR1

Add 7 µl to each tube

Digest 1¼ hrs. @ 37°C. Add 3 µl loading buffer/dye.

Run @ 55 volts on FMC Seakem 1% agarose (24 well format gel) in 1X TBE w/ Et Br.

Photo:



Friday 1 November 1996

Spin down Hu 1, ~~3~~, 4 plasmid preps + Rat 11 & 12
Resuspend in T.E. + make 1:100 dil for O.D.

Vol	Sample	260	280	260/280	Conc	Total
100	R-11	0.288	0.154	1.9	1.44	144 µg
100	R-12	0.182	0.098	1.9	0.91	91 µg
250	Hu 1	0.572	0.301	1.9	2.86	~~[illegible]~~ 715 µg
250	Hu 2	0.324	0.171	1.9	1.62	405 µg
250	Hu 4	0.557	0.290	1.9	2.79	696 µg

(Margin notes: R-11 and R-12 — "re-picks of 11 + 12"; R-12 circled — "thrown out 11/11/96 after seeing gel")

Monday 4 November 1996

Cut 10 µg Hu 4 for making probe to screen Human library:

1.5 µl DNA
32.5 µl dH_2O
4 µl 10X H buffer
2 µl EcoRI

40 µl

Add 10 µl dye

2 lanes

Run on LMP agarose gel w/ Marteen.
See next page for photo.

Monday 4 November 1996

Photo of LMP agarose gel:

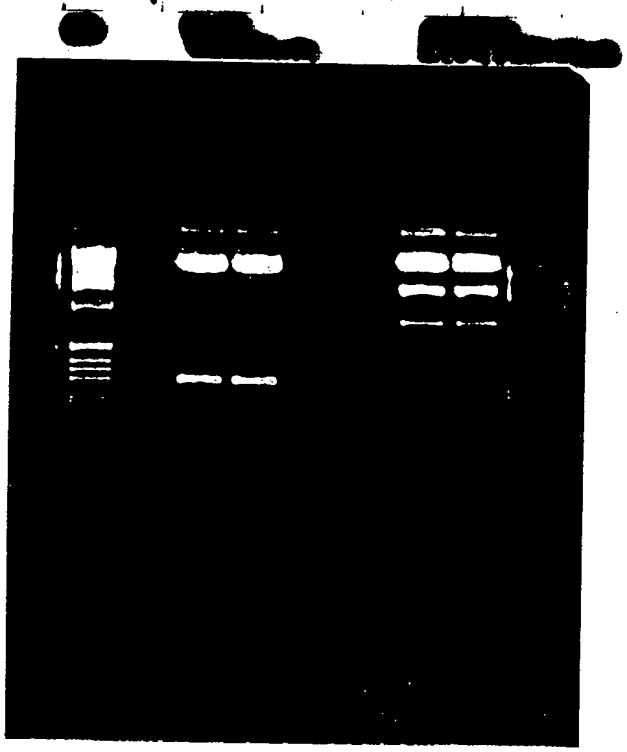
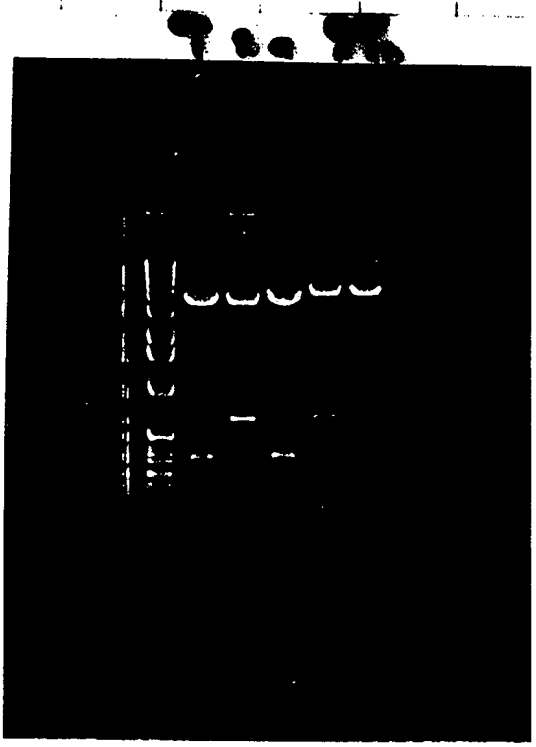


Photo of FMC gel:



Tare = 1.36

Hu4 = 1.45 - 1.36 = 90 µl

$$\frac{\frac{250}{4150} \times 10}{90} = \frac{602}{90} = 6.7 \text{ ng/µl}$$

Hu 1, 2, 4 = 0.5 µl DNA
Rat 11, 12 = 1.0 µl DNA
17.0 or 16.5 µl dH_2O
2.0 µl ~~BamHI~~ or ~~EcoR~~
0.5 µl BamHI or EcoR
20 µl

Use 7 µl for ~50 ng labeling aliquot.

NB: Rat 11 is probably contamination — 12 is the wrong size!

→ Label 6 x 50 ng @ 37°C for 30', 0/N @ R.T.

Monday 4 November 1996

Labeling: 7 µl DNA } x 6 → boil 8' → ↘ 42°C
25 µl dH_2O }

Add to each tube:
1 µl BSA (10 mg/ml)
10 µl 5XOLB
5 µl α^{32}P dCTP (3000 C:/mMol)
2 µl Klenow (2U/µl)

50 µl

30' @ 37°C, O/N @ RT.

Tuesday 5 November 1996

Heat probes @ 68°C for 10'.
Add 50 µl Ø-OH ⇔ 1XTNE + pre warmed.
Vortex. Spin.
Put aqueous phase over prepared G-50 probe quant Columns.

Count 2 µl of each labeled probe:

USER: 3 ID:32P COMMENTS:32P
PRESET TIME: 1.00 H#: NO SAMPLE REPEATS: 1 DATA CALC:
PRINTER:EDITED SCR:YES REPLICATES: 1 COUNT BLANK:
RS232: OFF RCM:YES MULTIPLIER: 1.000000 VIAL SIZE: MA

ISOTOPE 1: 32P %ERROR: 0.50 BKG. SUB: 0 HALF LIFE:YES

SAM NO	POS	TIME MIN	SCR	32P CPM	32P %ERROR	RCM	ELAPSED TIME
1	1-1	1.00	0.889	45.00	29.81	1.24	1.50
2	1-2	0.10	1.000	2922037.8	0.37	0.01	2.91 - Hu41
3	1-3	0.10	1.000	2983095.5	0.37	0.01	4.34 - 2
4	1-4	0.10	1.000	3035919.0	0.36	0.02	5.78 - 3
5	1-5	0.05	1.000	3593257.8	0.47	0.01	6.84 - 4
6	1-6	0.05	1.000	3280587.3	0.49	0.02	7.88 - 5
7	1-7	0.10	1.000	2746072.5	0.38	0.02	9.22 - 0

Monday 4 November 1996

Sequencing shows that Hu 1, 4, 5 are the correct fragment of Human contisfaction.

Pam "zapped" the human brain library in pT_7T_3D - Eco - NoT into sme cells & checked # of transformants on plates - She used 45 ng.

Tuesday 5 November 1996

10^{-1} dilution, 1 µl = 4000 colonies

Lurs spread plates @ ~ 6:30 pm & put in incubator - 25 plates

Wednesday 6 November 1996

Removed plates from incubator @ 9:45 am. Lifted onto Brotmans filters. Denatured, neutralized, air dry, crosslink, prewash & put filters into prehyb @ 12:30 pm. Std. Southern PH/H solution - 200 ml. Plates returned to incubator to "grow-out" @ ~ 11 am. Remove to RT & then cold room at _____ pm.

Lurs will add 4X probe (Hu 4) @ 7-8 pm tonight.

Thursday 7 November 1996

Wash library screening filters to 0.2 x SSC @ 68°C & put on film w/ 2 screens O/N.

Friday 8 November 1996

Develop films.

Results: 2 positives! - plate 8 & plate 24

Picked to 1ml LBAmp cultures - afternoon.

Luis spread several dilutions on small & large plates - he also lifted them, regrew the plates & probed the filters & washed on Monday 11/11/96. Probe used was 40 ml of the 200 ml (PH/H + 3 batches of probe) from primary screen of last week.

Monday 11 November 1996

Develop secondary/tertiary (skipped grid step since there were only 2 putatives positives!) screen film.

Start O/N cultures 3X 100 ml each of Hu 8 & Hu 24. Labeled: HuCort 8 & HuCort 24 & put away 1ml glycerol stock. (Picked 1 positive colony for 8 & 24 into 3 ml LBAmp @ 3pm. Grow until 5 pm. Put 700 µl into 300 µl glycerol & put 0.5 ml to each 100 ml LBAmp in 500 ml flasks & grow O/N.

Tuesday 12 November 1996

Std Qiagen tip 500 preps of 100 ml O/N cultures. To ppt ~~DNA~~ w/ isopropanol & then NH_4OAc + EtOH. Resuspend combining 3X#8 + 3X#24 in 1 ml each: 1:100 dil for O.

	260	280	260/280	Conc	Total
HuCort 8	0.329	0.176	1.9	1.645	1,645 µg
HuCort 24	0.241	0.130	1.9	1.205	1,205 µg

Aliquot 4 x 10 µg of each for sequencing

Tuesday 12 November 1996

Aliquot 2 x 1 µl of HuCart 8 + HuCart 24 for EcoRI - NotI digest:

1 µl DNA
8.8 µl dH_2O
1.2 µl 10 x H buffer
0.5 µl EcoRI
0.5 µl NotI
12 µl

yes! #8 is contamination!